Studies on the Biosynthesis of Corrinoids and Porphyrinoids. IV.[1] Biosynthesis of Chlorophyll in *Euglena gracilis*

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The biosynthetic pathway of chlorophyll-a formation in the photosynthetic phytoflagellate *Euglena gracilis* was investigated by administration of ^{2}H- and ^{13}C-labeled precursors. L-[1-^{13}C]Glutamate was incorporated into eight carbon atoms in the chlorophyll macrocycle *via* δ-aminolevulinic acid formed through the C-5 pathway. A low incorporation of D-[1-^{13}C]glutamate was also observed. [2-^{13}C]Glycine was preferentially incorporated into the methyl ester carbon of chlorophyll *via* the one-carbon metabolic pathway, but not *via* the Shemin pathway. Feeding experiments with ^{2}H-, and ^{2}H,^{13}C-labeled glycine yielded labeled chlorophylls, which were subjected to nuclear magnetic resonance and mass spectra analyses to obtain detailed information about the biosynthetic origin of the hydrogen atoms.

Keywords chlorophyll; biosynthesis; incorporation; D-[1-^{13}C]glutamate; L-[1-^{13}C]glutamate; [2-^{13}C]glycine; [$^{13}CH_3$]-methionine

Naturally occurring tetrapyrrole compounds such as vitamin B_{12}, chlorophyll and protoheme are biosynthesized from δ-aminolevulinic acid (ALA).[2] In previous studies of chlorophyll biosynthesis, two pathways were shown to exist for the formation of ALA: in photosynthetic bacteria, ALA is formed by the condensation of glycine and succinyl-coenzyme-A (CoA) catalyzed by ALA synthase (Shemin pathway),[3] while in higher plants and greening algae, ALA is derived from the entire carbon skeleton of glutamate or 2-oxoglutarate (C-5 pathway).[4] The taxonomically interesting organism *Euglena gracilis* is known to synthesize ALA by the C-5 pathway.[5]

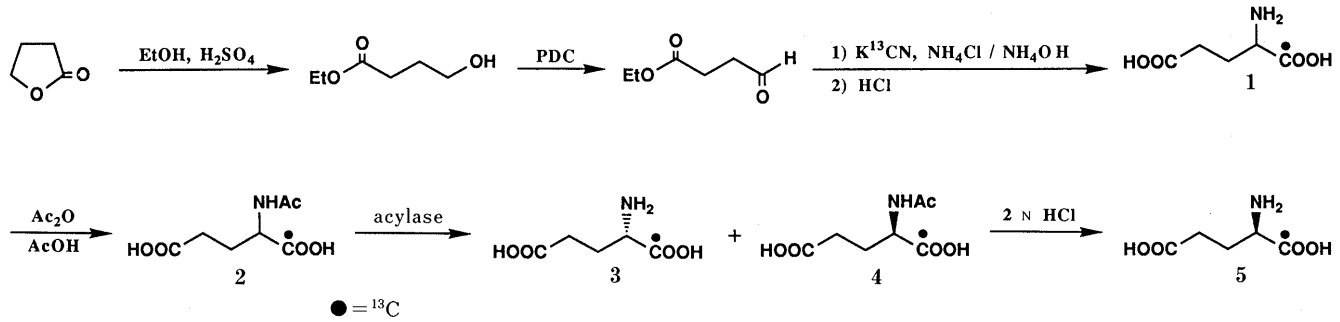
In 1982 Oh-hama *et al.*[6] reported that L-[1-^{13}C]glutamate is incorporated into chlorophyll *via* the C-5 pathway, while [2-^{13}C]glycine is not incorporated into the chlorophyll macrocycle *via* the Shemin pathway but is incorporated into the methoxyl group of the isocyclic ring E in cells of *Scendesmus obliquus*. Porra *et al.*[7] have reported similar results from feeding experiments with DL-[1-^{13}C]glutamate and [2-^{13}C]glycine in greening maize leaves. We now report the results of a further detailed investigation into the mechanism of the C-5 pathway and the biosynthetic origin of hydrogen atoms in the glycine metabolic sequence using the etiolated photosynthetic phytoflagellate *Euglena gracilis*.

Results and Discussion

The synthesis of L- and D-[1-^{13}C]glutamic acid is illustrated in Chart 1. An adaptation of Fotadar and Cowburm's method[8] was used to prepare DL-[1-^{13}C]glutamic acid (**1**). The acetylation of **1** with acetic anhydride and acetic acid gave DL-[1-^{13}C]*N*-acetylglutamic acid (**2**). This was hydrolyzed with porcine kidney acylase, which catalyzes the hydrolysis of *N*-acetyl L-α-amino acids[9]; kinetic resolution of the enantiomers occurred to give L-[1-^{13}C]glutamic acid (**3**) and D-[1-^{13}C]*N*-acetylglutamic acid (**4**) in 36% and 43% yields, respectively. After separation, **4** was hydrolyzed with 2 N HCl to give D-[1-^{13}C]glutamic acid (**5**). The optical purities of **3** and **5** were determined by high performance liquid chromatography (HPLC) using a chiral column to be 100% ee and 98.8% ee, respectively.

Labeled glycine[10] and methionine[11] were synthesized by the literature procedures from labeled sodium acetate and methyl iodide.

A feeding experiment was carried out with etiolated *Euglena gracilis*. After addition of labeled precursors, cells were cultured photosynthetically under light for 4 d, and the produced chlorophyll was extracted and purified. The ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectra of chlorophyll derived from the feeding experiments with L- and D-[1-^{13}C]glutamate are shown in Fig. 1 and signal assignments are also illustrated, based on early studies.[6,7,12] The spectra clearly show ^{13}C enrichment of eight carbons at 92.3, 99.8, 103.8, 107.6, 146.0, 148.5, 155.1 and 161.7 ppm (δ, α, γ, β, C-14, -12, -16 and -17, respectively) by comparison with the natural abundance intensities. The



acylase reaction
conditions : acylase (porcine kidney), 2.6 mg (2340 units); substrate, 250 mg/5 ml water (pH 7.2); temperature, 37°C; reaction time; 7 h

Chart 1. Synthesis of L- and D-[1-^{13}C]Glutamic Acid

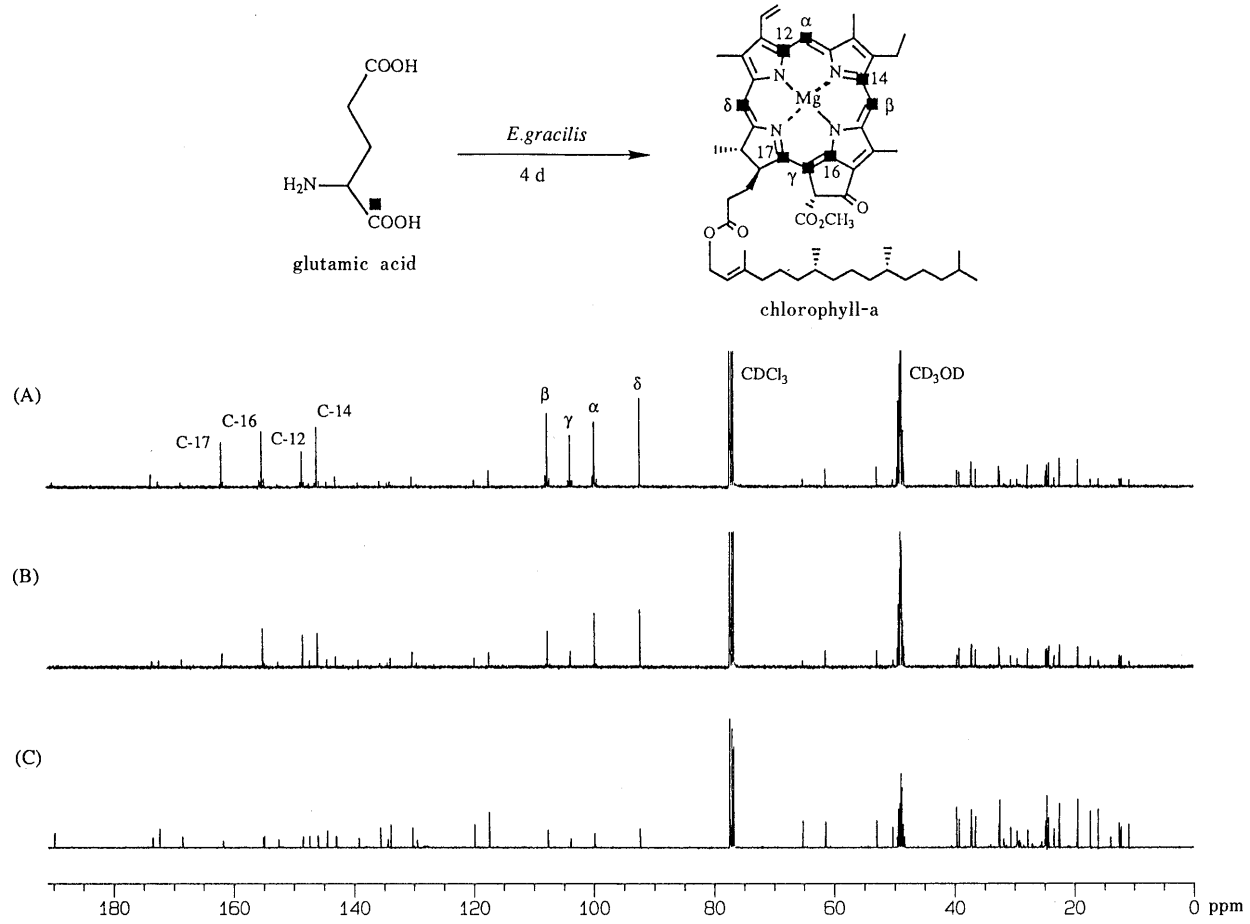


Fig. 1. Comparison of the ^{13}C-NMR Spectra (Solvent $CDCl_3:CD_3OD=88:12$) of L-[1-^{13}C]Glutamate-Incorporated Chlorophyll-a (A) and D-[1-^{13}C]Glutamate-Incorporated Chlorophyll-a (B) with the Natural Abundance Spectrum (C)

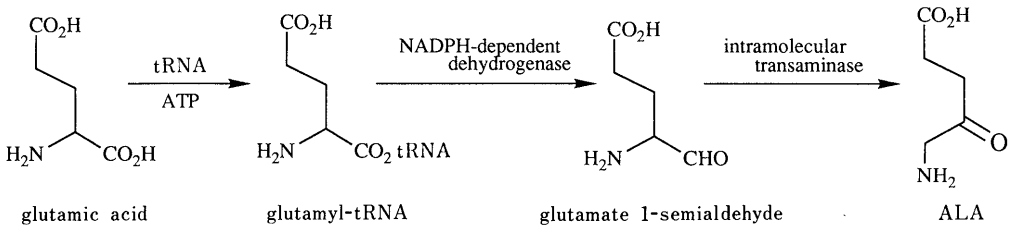


Chart 2. Biosynthesis of ALA from Glutamate

data showed that both L- and D-[1-^{13}C]glutamate were incorporated into chlorophyll *via* [5-^{13}C]ALA through the C-5 pathway. In the L-[1-^{13}C]glutamate feeding experiment, signals of the ^{13}C-enriched carbon atoms were split into doublets owing to ^{13}C–^{13}C spin coupling with adjacent ^{13}C atoms, except for δ carbons. Comparison of the integrated intensities of the singlet and doublet resonances indicated a 17—21% enrichment at each center, which corresponded to an approximately 14% incorporation. However, the D-[1-^{13}C]glutamate feeding experiment did not give such ^{13}C–^{13}C spin coupling so that the extent of incorporation could not have been more than about 5%.

The labeled chlorophylls were then converted into the corresponding more stable methyl pheophorbides by treatment with 0.5% sulfuric acid in methanol. The expanded ^{13}C-NMR spectra of the labeled methyl pheophorbides are shown in Fig. 2. The ^{13}C enrichments are numerically almost equal to those described above.

The published sequence of the C-5 pathway from glutamate to ALA is shown in Chart 2.[14] Our results indicate that the specificity of the glutamyl-transfer ribonucleic acid (tRNA) synthase toward L-glutamate is high. The results also suggest that if glutamyl-tRNA synthase is active towards only L-glutamate, D-glutamate must have been transformed into L-glutamate by glutamate racemase and then metabolized to ALA through the C-5 pathway.

Next, an incorporation experiment with [2-^{13}C]glycine showed no enrichment at the eight carbons expected from the Shemin pathway, though the enzyme involved (ALA synthase) is known to operate in *Euglena gracilis*,[15] but ^{13}C enrichment was observed at the C-10b methyl carbon (52.9 ppm). The methyl carbon would be derived by C-methylation *via* *S*-adenosylmethionine (SAM). [Methyl-^{13}C]methionine was subsequently incorporated into chlorophyll under identical conditions. The ^{13}C-NMR spectrum showed the highly enriched C-10b resonance (a 4-

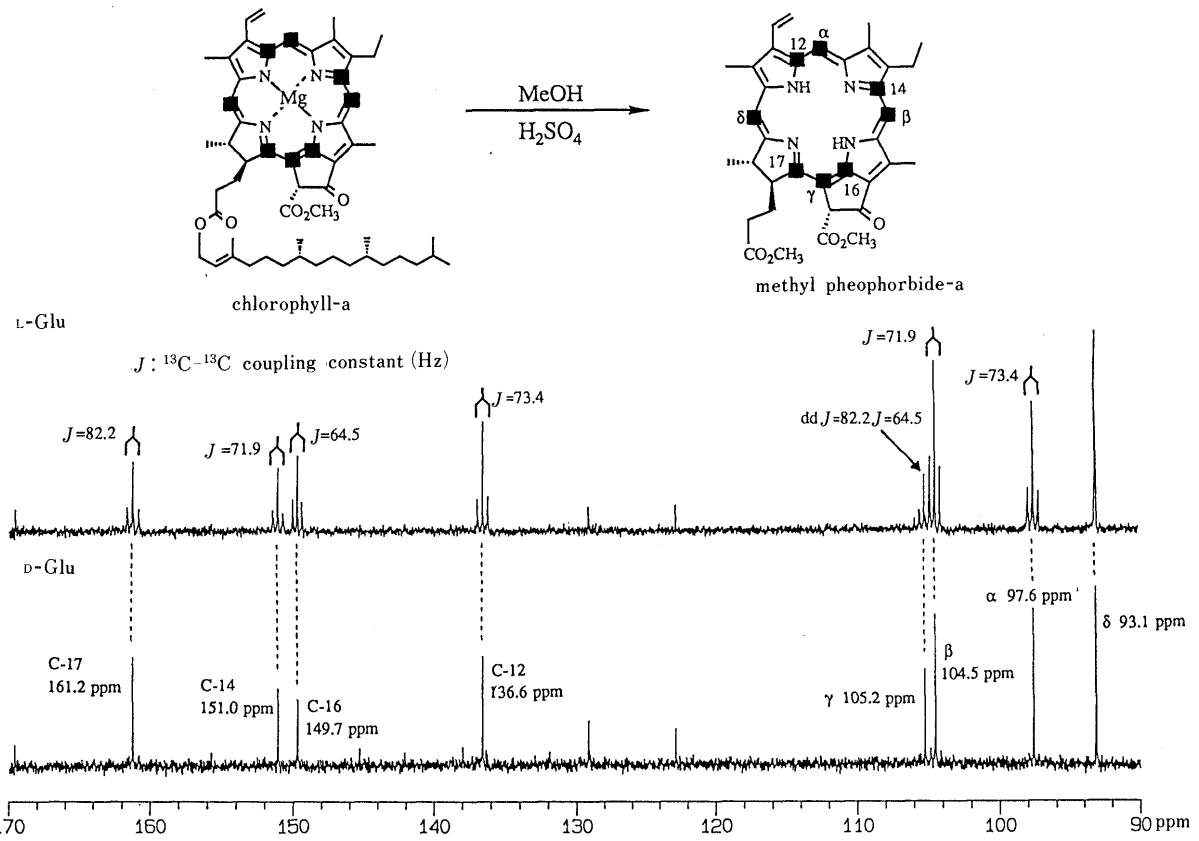


Fig. 2. Comparison of the Expanded ^{13}C-NMR Spectrum (Solvent $CDCl_3$) of L-[1-^{13}C]Glutamate-Incorporated Methyl Pheophorbide-a (Above) with That of D-[1-^{13}C]Glutamate-Incorporated Methyl Pheophorbide-a (Below)

The assignments of enriched signals were made in accordance with reported data.[13]

fold enhancement of the C-10b signal was observed by comparison with the [2-^{13}C]glycine feeding experiment).

These biosynthetic results are similar to those reported previously.[6,7] Glycine can act as a one-carbon donor by carbon transfer to tetrahydrofolate which can then transfer the carbon to homocysteine to form methionine, which is a versatile carbon donor. In this process the C-2 of glycine may be transformed into methylene of N^5,N^{10}-methylene-tetrahydrofolate either directly by glycine synthase or indirectly *via* the C-3 of serine by serine hydroxymethyl-transferase. These enzyme reactions have been found in mitochondria[16] and chloroplasts.[17] In photosynthetic bacteria (*Rhodopseudomonas spheroides* S), C-2 of glycine can be transformed both *via* the Shemin pathway and *via* the above one-carbon metabolic pathway into bacterio-chlorophyll.[18] The results of our investigation indicated that C-2 of glycine was preferentially incorporated into the methyl ester carbon of chlorophyll, but not through the Shemin pathway.

Additional data on the fate of substrate hydrogens came from the incorporation of deuterium-labeled glycine, helping to define the biosynthetic origins of the hydrogen atoms and the nature of the biosynthetic mechanisms. A feeding experiment with [2-^{13}C,2-$^{2}H_2$]glycine gave chlorophyll whose ^{13}C-NMR spectrum showed the signals of ^{13}C-enriched carbons as singlets. This implies a loss of the deuterium atoms in the biosynthetic pathway. To investigate this further, pulsed feeding experiments with [2-$^{2}H_2$]glycine was carried out, and the labeled chlorophyll was subjected to fast atom bombardment (FAB)-mass

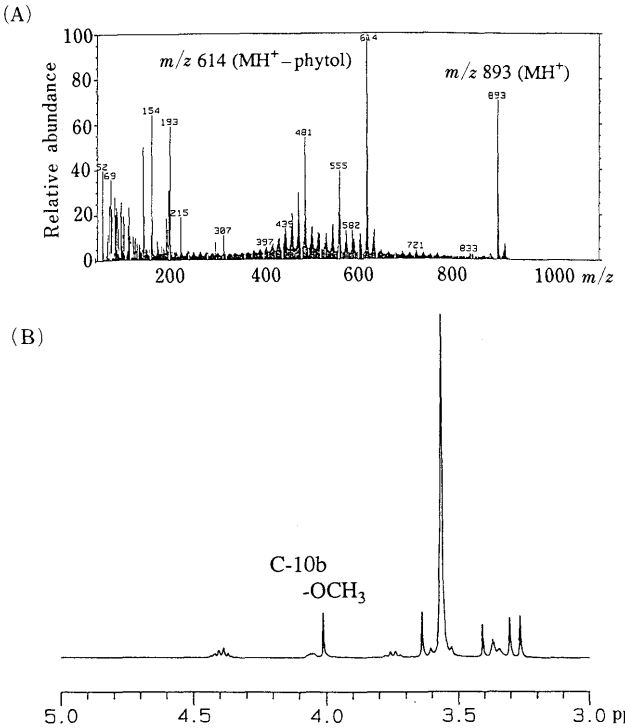


Fig. 3. FAB-MS (A) and ^1H-NMR Spectra (Solvent $CDCl_3$: CD_3OD = 88 : 12) (B) of [2-$^{2}H_2$]Glycine-Incorporated Chlorophyll-a

spectral analysis. Prominent *m/z* 893 (MH^+) and 614 (MH^+ – phytol) peaks were observed, indicating a total loss of deuterium atoms (Fig. 3-A). The ^1H-NMR spectrum is

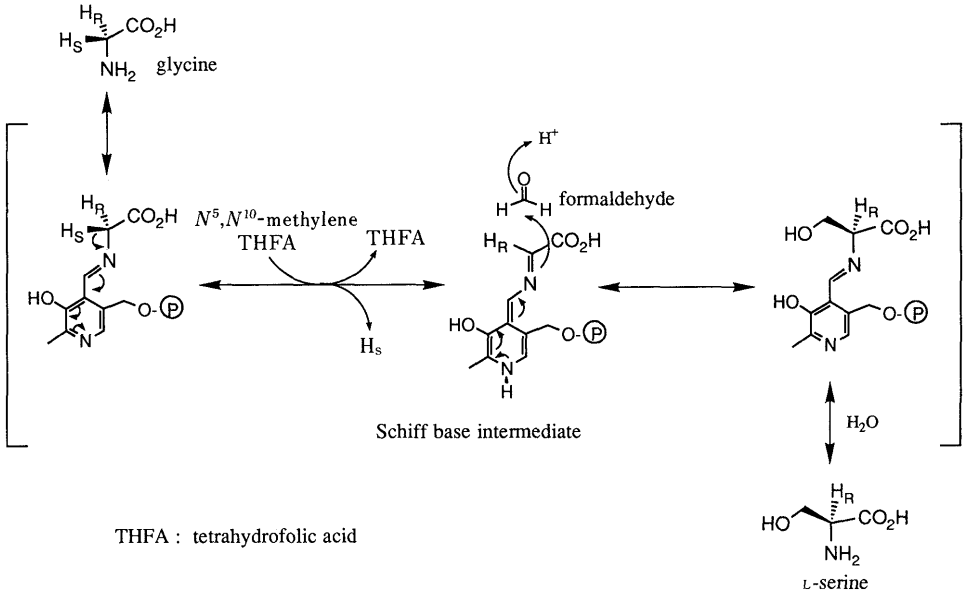


Chart 3. A Mechanism for the Serine Hydroxymethyltransferase-Catalysed Interconversion of Glycine and Serine

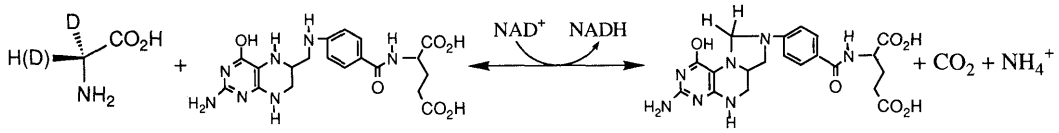


Chart 4. Reaction Catalyzed by Glycine Synthase

also shown in Fig. 3-B. The signal of the C-10b methyl protons (4.0 ppm) was detected as a singlet of about 3H intensity. The spectrum was consistent with the results of FAB-mass analysis. From the data presented here, it is clear that the biosynthesis of SAM from glycine involved total loss of the deuterium atoms in [2-$^2H_2$]glycine.

The mechanism by which serine hydroxymethyltransferase catalyzes the interconversion of glycine and serine is shown in Chart 3.[19,20] When the glycine-pyridoxal phosphate Schiff base is formed, the enantiotopic *pro-S* hydrogen atom of glycine undergoes stereospecific exchange with a proton of the solvent.[21,22] Our results indicate that the *pro-R* hydrogen atom of glycine was also exchanged in another stage. We suggest that this occurred in the reaction catalyzed by glycine synthase (Chart 4). Thus, when glycine was incorporated into the methyl ester carbon of chlorophyll, the protons at C-2 of glycine were completely exchanged during these enzyme reactions. We concluded that two of the hydrogen atoms of the C-10b methyl group are derived from the medium. The remaining one hydrogen is derived from hydride, generated from reduced nicotinamide adenine dinucleotide (NADH) (N^5,-N^{10}-methylenetetrahydrofolate is reduced to N^5-methyltetrahydrofolate by NADH, and N^5-methyltetrahydrofolate then reacts with homocysteine, to produce methionine).

Experimental

All melting points were taken on a Yanaco hot stage microscope apparatus type MP-500D and are uncorrected. Infrared (IR) spectra were recorded on a JASCO FT-IR 5000 spectrometer. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL GSX-400 spectrometer (400 and 100 MHz). Chemical shifts are given downfield from tetramethylsilane (TMS) or sodium 3-trimethylpropionate-d_4 (TSP) in the case of ^{1}H-NMR, and from chloroform-d_1 (=77.0 ppm) or dioxane (=67.4 ppm) as an internal standard in the case of ^{13}C-NMR. Mass spectra (MS) were recorded on a JEOL DX-302 spectrometer equipped with a JMA DA-5000 data system. The matrix used was 3-nitrobenzyl alcohol (3-NOBA) in the case of FAB-MS. Ultraviolet (UV) spectra were recorded on a JASCO UVIDEC-610C spectrometer. HPLC was performed using a JASCO LC-800 chromatography system.

Labeled Glycine [2-^{2}H]Glycine was obtained from Merck of Canada and used as received (98 atom % ^{2}H), while [2-^{13}C]- and [2-^{13}C,2-$^2H_2$]glycine were synthesized by literature procedures[10] from [2-^{13}C]-sodium acetate (99 atom % ^{13}C, 40.0% yield for 3 steps) and [2-^{13}C,2-$^2H_3$]sodium acetate (99 atom % ^{13}C; 98 atom % ^{2}H, 36.6% yield for 3 steps), respectively.

L-[Methyl-^{13}C]methionine L-[Methyl-^{13}C]methionine was synthesized by literature procedures[11] from [^{13}C]methyl iodide (92 atom % ^{13}C, 78.0% yield).

DL-[1-^{13}C]Glutamic Acid (1) DL-[1-^{13}C]Glutamic acid was synthesized by literature procedures[8] from potassium [^{13}C]cyanide (90 atom % ^{13}C, 87.1% yield).

DL-[1-^{13}C]*N*-Acetylglutamic Acid (2) Acetic anhydride (4.5 ml) was added to a solution of **1** (1.04 g, 7.0 mmol) in acetic acid (12 ml), and the mixture was stirred at 110 °C for 5 min. After evaporation of the acetic anhydride and acetic acid, water was added to the residue. Freeze-drying gave **2** (1.18 g, 88.1%), mp 184.1—186.6 °C. ^{1}H-NMR (D_2O) δ: 2.03, 2.24 (each 1H, m, $-C\underline{H}_2CH_2COOH$), 2.04 (3H, s, $-NHCOC\underline{H}_3$), 2.53 (2H, t, J=7.2 Hz, $-CH_2C\underline{H}_2COOH$), 4.43 (1H, ddd, $^2J_{^{13}C\text{-}H}$=3.3 Hz, J=5.6 Hz, J=9.0 Hz, $-C\underline{H}^{13}COOH$). ^{13}C-NMR (D_2O) δ: 175.01 (C-1). IR (KBr) cm^{-1}: 3500—2900 (br, -OH, $-NH_3^+$), 1709, 1675 (s, C=O), 1570, 1560 (m, $-NH_3^+$). MS *m/z*: 190 (M^+).

L-[1-^{13}C]Glutamic Acid (3) Compound **2** (250 mg, 1.3 mmol) was added to distilled water (5 ml) then the pH was adjusted to 7.2 with aqueous ammonia, and 2.6 mg of acylase I (Sigma Grade II: from porcine kidney, 900 units/mg) was added. The mixture was stirred at 37 °C for 7 h, then heated to 60 °C with Norit for 3 min, and filtered. The filtrate was applied to a column (2.5 × 10 cm) of Dowex-50 (H^+ form), and the column was washed with distilled water (300 ml) and then eluted with 1 N aqueous ammonia (260 ml). The aqueous ammonia layer was freeze-dried and the

residue was dissolved in water. The solution was adjusted with aqueous ammonia to pH 3. The precipitate was collected by filtration to give **3** (70.2 mg, 36.0%), mp 205.8—206.8 °C. ^{1}H-NMR (D_2O) δ: 2.17 (2H, m, $-C\underline{H}_2CH_2COOH$), 2.57 (2H, m, $-CH_2C\underline{H}_2COOH$), 3.83 (1H, dt, $^2J_{^{13}C\text{-}H}=5.14$ Hz, $J=6.16$ Hz, $-C\underline{H}^{13}COOH$). ^{13}C-NMR (D_2O) δ: 175.32 (C-1). IR (KBr) cm^{-1}: 3330—2830 (br, –OH, $-NH_3^+$), 1686 (w, C=O), 1560, 1543, 1510 (s, $-NH_3^+$). MS *m/z*: 130 (M^+-H_2O).

D-[1-^{13}C]Glutamic Acid (5) The above distilled water eluate (300 ml) was freeze-dried to give crude **4**, which was dissolved in 2 N HCl (12 ml). The mixture was stirred at 110 °C for 3.5 h, then freeze-dried. The residue was dissolved in water and the solution was adjusted with aqueous ammonia to pH 3. The precipitate was collected by filtration to give **5** (83.9 mg, 43.1%), mp 200.5—203.5 °C. ^{1}H-NMR (D_2O) δ: 2.17 (2H, m, $-C\underline{H}_2CH_2COOH$), 2.57 (2H, m, $-CH_2C\underline{H}_2COOH$), 3.83 (1H, dt, $^2J_{^{13}C\text{-}H}=5.65$ Hz, $J=6.16$ Hz, $-C\underline{H}^{13}COOH$). ^{13}C-NMR (D_2O) δ: 175.32 (C-1). MS *m/z*: 130 (M^+-H_2O).

The optical purity of **3** and **5** was examined by HPLC using a chiral column (4.0 mm i.d. × 150 mm) packed with Crownpac CR(−). The mobile phase used was distilled water adjusted to pH 1.5 with $HClO_4$. A UV detector set at 200 nm was used for detection. The flow rate was maintained at 0.4 ml/min throughout.

Culture Conditions *Euglena gracilis* IME E-3 was grown photosynthetically in the light (100 W × 2) at 30 °C in a static culture, each flask holding 10 ml of an aqueous solution containing L-glutamic acid (5 g), DL-malic acid (2 g), L-methionine (50 mg), thiamine hydrochloride (1 mg), cyanocobalamin (5 μg), KH_2PO_4 (400 mg), $(NH_4)_2PO_4$ (200 mg), $CaCO_3$ (100 mg), $ZnCl_2$ (10 mg), H_3BO_4 (80 μg), ethylenediaminetetraacetic acid (EDTA) (10 mg), $MgSO_4\cdot 7H_2O$ (500 mg), $FeSO_4\cdot 7H_2O$ (4 mg), $CoCl_2\cdot 6H_2O$ (2 mg), $MnCl_2\cdot 4H_2O$ (2 mg), and $CuCl_2\cdot 2H_2O$ (400 μg) per liter of distilled water. Preparation of etiolated cells of *Euglena gracilis* was done by culture in the dark at 30 °C for 4 d in the above medium.

Incorporation of D- and L-[1-^{13}C]Glutamic Acid Four-day-cultured etiolated cells (10 ml × 4) were resuspended in 100 ml of medium containing [1-^{13}C]glutamic acid (60 mg). The medium was cultured photosynthetically in the light for 4 d. The cells were gathered, washed with brine, and disrupted with an ultrasonicator (NIC US-300) at 0 °C for 10 min in methanol solution. The suspension was centrifuged at 10000 rpm at 4 °C for 15 min. The supernatant was evaporated to dryness. The residue was pre-purified by column chromatography (ODS, 2.5 i.d. × 25 cm, methanol). The green fraction was collected, evaporated and further purified by HPLC (ODS, 5 μm, 1.5 i.d. × 25 cm, water–methanol) to give 2 mg of pure chlorophyll-a.

Incorporation of [2-^{13}C]- and [2-^{13}C,2-^{2}H$_2$]Glycine Feeding conditions were exactly the same as those for glutamate incorporation.

Incorporation of L-[Methyl-^{13}C]methionine The same procedure as above was used except that the amount of L-[methyl-^{13}C]methionine in 100 ml of medium was 50 mg.

Conversion of ^{13}C-Labeled Chlorophyll to Methyl Pheophorbide Chlorophyll labeled by feeding [1-^{13}C]glutamate was dissolved in 0.5% sulfuric acid in methanol (10 ml) and left at room temperature for 12 h. Dilution with CH_2Cl_2 (200 ml), followed by washing with saturated $NaHCO_3$ solution (2 × 200 ml), water (200 ml) and brine (200 ml), drying (Na_2SO_4), and concentration, gave a residue which was purified by HPLC (silica, 5 μm, 1.0 i.d. × 25 cm, hexane : isopropanol = 95 : 5) to give 1.2 mg of pure labeled methyl pheophorbide-a.

Pulsed Feeding Experiments with [2-^{2}H$_2$]Glycine Four-day-cultured etiolated cells (10 ml × 4) were resuspended in 100 ml of medium containing [2-^{2}H$_2$]glycine (30 mg) and cultured photosynthetically in the light. On each of 4 succeeding days, 10 mg of [2-^{2}H$_2$]glycine was dissolved in 1 ml of water and added to the feeding medium. After 4 d, chlorophyll produced was extracted and purified according to the general procedure.

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